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Effects of overexpression of antioxidants on the release of cytochrome *c* and apoptosis-inducing factor in the model of ischemia

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ABSTRACT

Apoptosis arises from neuronal damage following an ischemic insult. Apoptosis-inducing factor (AIF) is a protein released from mitochondria in response to pro-apoptotic signals which then translocates to the nucleus and triggers DNA fragmentation. In parallel with this, pro-apoptotic signals cause the release of cytochrome *c* from mitochondria, activating caspase-dependent apoptosis. During post-ischemic reperfusion, reactive oxygen species (ROS) are formed in excess in mitochondria and can play a role in initiating apoptosis. In cultures, ROS are formed during post oxygen glucose deprivation (OGD) normoxia/normoglycemia that is used as a model for ischemia. In this study, we delivered viral vectors to overexpress antioxidants (GPX, catalase, CuZnSOD, or MnSOD) in mixed cortical cultures, in order to investigate the effects of ROS-reduction on the release of cytochrome *c* and AIF. Overexpression of MnSOD, CuZnSOD, catalase or GPX all prevented AIF translocation from mitochondria to the nucleus. Potentially, this could reflect broadly non-specific protection due to reducing ROS load. Arguing against this, overexpression of the same antioxidants did not inhibit cytochrome *c* release. These findings suggest a specific interaction between ROS formation and the caspase-independent route of apoptosis.

Cerebral ischemia begets an energy crisis that is exacerbated by increased synaptic glutamate concentrations. This build up leads to excessive calcium influx in the post-synaptic neuron that, once reperfusion occurs, causes the generation of highly damaging reactive oxygen species (ROS) [10]. As a defense against this, neurons express endogenous antioxidants which detoxify ROS. Superoxide anion is converted to hydrogen peroxide by superoxide dismutase (SOD), which exists as MnSOD in mitochondria and as CuZnSOD in cytosol. The localization of these SOD subtypes is most likely associated with different degrees of protection against superoxide that is produced primarily in the mitochondria. Hydrogen peroxide is then detoxified to water and oxygen by glutathione peroxidase (GPX) or catalase (Cat).

Though necrosis characterizes most neuron death post-ischemia, death in the penumbra region is mostly apoptotic and has been hypothesized to have the greatest potential for effective treatment. Two crucial mitochondrial proteins, cytochrome *c* and apoptosis-inducing factor (AIF) are released from mitochondria during ischemia [19]. Once in the cytosol, cytochrome *c* activates the caspase cascade, resulting in many of the facets of apoptosis [2]. In contrast, AIF translocates directly to the nucleus, where it causes the DNA fragmentation typical of apoptosis [12].

The release of AIF can occur independently from cytochrome *c* release and caspase activation [9], leading to its characterization as "caspase-independent." However, recent studies suggest potential cross-talk between caspase-dependent and independent pathways, since caspases and the caspase-activated protein t-Bid can, in fact, trigger the release of AIF from the mitochondria [4].

While reducing ROS levels can inhibit apoptosis, the underlying mechanisms are not fully understood. ROS can damage the mitochondrial membrane, compromise mitochondrial membrane potential, and induce cytochrome *c* release [6]. In contrast, the direct link between ROS generation and the release of AIF is less clear.

To this end, in this study, we use herpes simples virus-1 (HSV-1) vectors overexpressing GPX, catalase, CuZnSOD, or MnSOD in mixed cortical cultures, in order to investigate the effects of ROS-reduction on the release of cytochrome *c* and AIF. GPX, catalase and CuZnSOD have been previously shown to be neuroprotective in the model of ischemia [21,23].

Cortical cultures were prepared from day 18 fetal rats by standard culturing techniques [1]. Cortical tissue was treated with papain (Worthington, Lakewood, NJ) and subsequently dissociated through an 80 μm cell strainer and resuspended in modified MEM media (Tissue Culture Facility UCSF, CA) supplemented with 10% horse serum (HyClone, Logan, UT). Cells were plated at a density of 1.2×10^5 cells/cm^2 on coverslips coated with poly-D-lysine (Sigma, St. Louis, MO). Under these conditions, cultures are approximately

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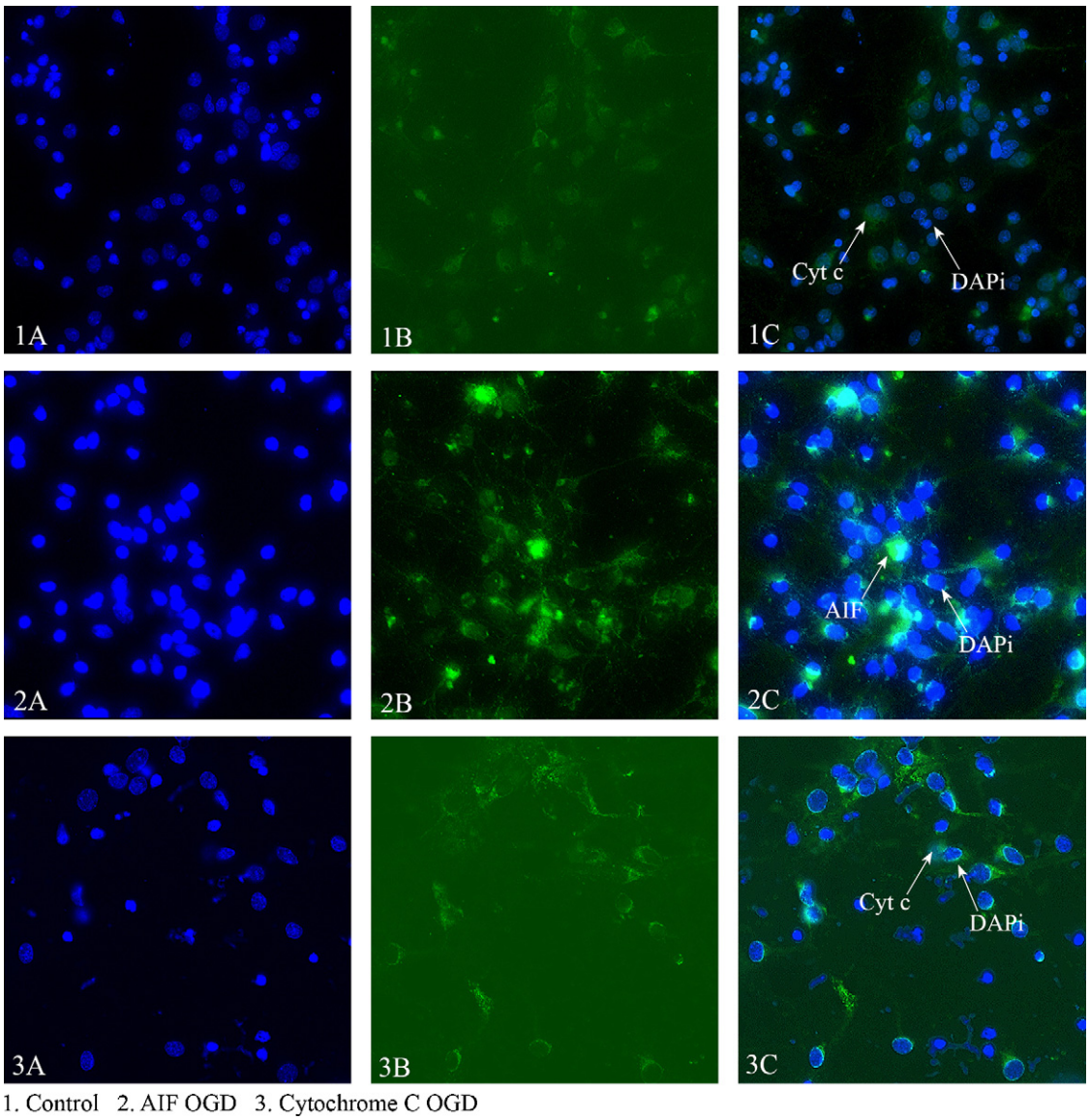

Fig. 1. Representative images of cytochrome *c* and AIF staining in cortical cultures showing how calculations were performed to determine the amount of cytochrome *c* and AIF in cells. (1A) cytochrome *c* staining in cytoplasm of control cultures not treated with OGD, (1B) DAPI staining of the nuclei, (1C) cytochrome *c* staining overlaid with DAPI staining, (2A) AIF staining in OGD treated cultures, (2B) DAPI staining of nuclei, (2C) AIF staining overlaid with DAPI staining, (3A) cytochrome *c* staining in cytoplasm of OGD treated cultures, (3B) DAPI staining of nuclei, (3C) cytochrome *c* staining overlaid with DAPI staining.

20–30% neuronal [15]. Experiments (described below) were conducted on days 10–12 of culturing.

Herpes simplex virus-1 amplicon vectors containing Bgal or GFP as a reporter gene and the gene for one of the antioxidants–CuZnSOD, MnSOD, GPX or Catalase were constructed by methods established in this laboratory [15]. Control vectors expressed Bgal or GFP alone. Two different reporter genes were used, but we proved from the work in the laboratory that there is no difference between these control vectors. Constructed plasmids were inserted into HSV-1 amplicon by standard methods [5]. Viral titers were approximately $0.8–2 \times 10^7$ virus particles/mL.

Cells on coverslips were infected with 10,000 viral particles/coverslip (constant MOI of 0.03) with the indicated vector preparations as previously established in the laboratory [21]. Under these conditions, 45% of neurons and 5% of glia were infected [15]. 18–24 h later cells were exposed to oxygen/glucose deprivation (oxygen glucose deprivation (OGD)-treated cultures) by replacement of cell media with 0 mM glucose MEM (made in-house) and incubation in an anaerobic environment of 90% nitrogen, 5% CO_2 and 5% hydrogen for 3 h followed by 3 h of normoxic/normoglycemic conditions before fixing the cultures in cold methanol. In control cultures, media was changed to MEM containing 5 mM glucose and left in normoxic conditions. At these times points no cells death was occurring. There was no significant difference in the number of cells between the control and experimental wells (data not shown). A minimum of three different weeks of cell cultures were used in each experiment.

Cold methanol-fixed cultures were treated with Triton X (0.2% in PBS) for 30 min and blocked with 3% bovine albumin serum in Triton X (0.2% in PBS) for 30 min before addition of primary anti-cytochrome *c* antibody (1/200 dilution) (BD Pharmingen, San Jose, CA) in 0.1% Triton X/1% BSA in PBS for 24 h. Secondary biotinylated antigoat antibody was conjugated to FITC (1/200 dilution) (Vector, Burlingame, CA) and added in 0.1% Triton X/1% BSA in PBS for 18 h. Nuclei were visualized with DAPI staining.

Cold methanol-fixed cultures were blocked in 5% milk and 5% rabbit serum in PBS (blocking solution) for 1 h and then treated with Triton X (0.2% in PBS) for 10 min before addition of the